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Comparison of different methods of analysis of vitamin B12 in fortified food and beverage samples

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Introduction

- Increasing awareness about the importance of water-soluble vitamins in preventive health care is expanding the customer base for the vitamin-fortified foods worldwide.
- Vitamin B12 fortification is now receiving wider attention because of its potential deficiency in different segments of population and also in those with restricted intake of animal based foods. Proper amount of vitamin B12 is required to take full advantage of folate fortification.

Introduction – Vit B12 Fortification

- **A wide variety of food, beverage and supplement products fortified with vitamin B12 are now manufactured worldwide to meet growing public demand.**
- **An increasing number of foods are being enriched with vitamins**
  - Different types of beverages (i.e., sports drinks, energy beverages, vitamin-fortified Cola)
  - Cereals
  - Various break fast items
  - Desserts

Introduction – Need for Rapid Assays

- **The vitamin contents of foods particularly those fortified are required to be monitored for product development, quality control and compliance purposes.**
- **The most commonly used methods for this purpose are microbiological**
  - Employed methods are generally lengthy, tedious, time consuming and poor in precision
- **HPLC methods are good but may need more sensitivity and specificity in case of vitamin B12.**
- **There is an urgent need for reliable rapid assays for water-soluble vitamins particularly vitamin B12.**

Introduction – Available Rapid Methods

- The conventional Microbiological methods have been made easier and relatively rapid by R. Biopharm's VitaFast Kit.
- Vit B12 Enzyme immunoassay for food and beverages is a simple and rapid method.
- Biacore's Biomolecular Interaction based assays (BIA) for B vitamins are rapid, accurate, precise, easy to use and more automated.
- The sensitivity and specificity of the LC methods can be improved by purifying and concentrating beverage or the food extract by immunoaffinity column.
- Lately LC-MS/MS methods have been applied for routine quality controls and product development purposes.

Objective

- Compare performance of different methods of vitamin B12 analysis in selected beverage samples.
- Methods compared: AOAC Microbiological, Vita Fast Micro method, ELISA method, Biosensor method and HPLC method.

  In selected samples comparison between Mico and LC-MS/MS methods.
- Performance evaluation of different methods for selected other matrices.

Methods

- **Conventional Microbiological method – FDA 410; AOAC 952.20 using *Lactbacillus leichmanni.***
- **Biosensor Method employed Biacore Q Biosensor system using vitamin B12 Qflex kit.**
- **Biopharm's VitaFast Vitamin B12 Method – Microbiological assay in microtiter plate kit format.**
- **R. Biopharm's Vitamin B12 Enzyme Immunoassay using ELISA microtiter plate**
- **HPLC method using R.Biopharm's Easy Extract Vitamin B12 immunoaffiniy column for purification and concentration of vitamin B12 in the sample extract or beverage samples.**

Extraction – Biosensor Method

- **Extraction Buffer: 0.09 M Na2HPO4, Citric acid 0.055M, NaCN 0.005M, pH 4.5**
- **Extraction: NIST SRM 1846 (Infant Formula) – 2g extracted in the 50 mL buffer by autoclaving for 15 min at 120°C.**
- **Beverages: Diluted 10x with extraction buffer containing bovine serum albumin.**

Vitamin B12 HPLC after Immunoaffinity Purification

- Extraction from NIST SRM 1846 (Infant Formula) by enzyme treatment with Pepsin and alpha-amylase in 50 mM sodium acetate pH 4.0 containing 1mL of 2% KCN
- Beverages adjusted to pH 7
- 5 - 10 mL sample passed through immunoaffinity column. Eluted in MeOH.
- Evaporated – taken in 0.025% TFA pH 2.6 (mobile phase).
- HPLC – Column C18 ACE aqueous 3µ, 3 x 150 mm
- Column Temp: 30°C
- Mobile Phase: Gradient; 0.025% TFA pH 2.6 & Acetonitrile
- UV, 361 nm; Inj: 100µL

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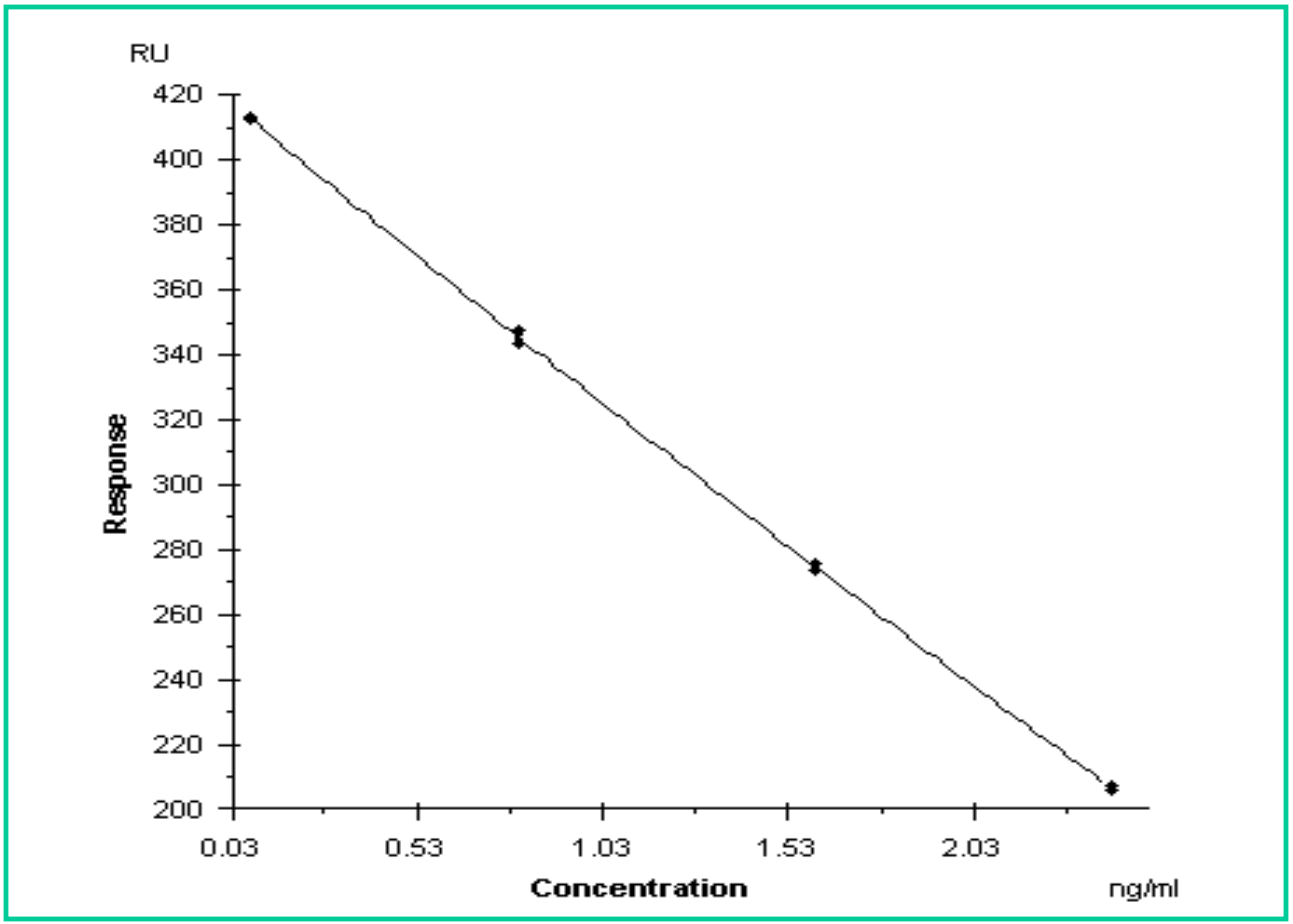
Biosensor Analysis - Vitamin B_{12} Calibration



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Vitamin B12 Biosensor Analysis in NIST SRM 1846 (Infant Formula) –Extraction Evaluation

NIST 1846 Infant Formula	Autoclave Time Temp		Results
	Time min	Temp F	mcg/100g
NIST 10, 226	10	226	4.19
NIST 15, 252	15	252	4.16
NIST 45, 252	45	252	3.88

Expected Vit B12 in NIST 1846 = 3.9 $\pm$ 0.3 mcg/100g

2 g of the sample extracted in 50 mL extraction solution.

Biosensor Analysis in NIST SRM 1846 (Infant Formula) -Accuracy & Precision

Parameter	mcg/100g
Mean	4.10
SD	0.39
% CV	9.6
n	8
Expected	3.9 ± 0.3

Biosensor Analysis in Beverages – Extraction Evaluation

Biosensor Method Extraction and processing treatments				Micro Method Results
BSA*	Yes	No	Yes	
Autoclave**	No	No	Yes	
Sample	Vitamin B12 mcg/100 mL			
Energy Bev 1	0.389	0.427	0.467	0.37
Energy Bev 2	0.296	0.39	0.534	0.33

* addition of bovine serum albumin in dilution (10 x) of the extract.

** Autoclave for 15 min at 252 F.

Biosensor Analysis in Beverages – Spike Recovery

Replicate	Vitamin B12 mcg/100g		Recovery
	Expected	Obtained	%
A	0.67	0.72	108
B	0.67	0.71	106
C	0.67	0.64	96
		Mean	103
		SD	6.7
		%RSD	6.4

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Vitamin B12 Analysis in Beverages by Biosensor Method – Precision

Crystal Light Energy Bevearge	Vitamin B12 (mcg/100mL) by Biacore Method			
	Mean	SD	% RSD	n
Sample 1	0.48	0.07	14.5	3
Sample 2	0.45	0.03	5.6	3
Sample 3	0.47	0.09	19.5	15
Sample 4	0.42	0.09	21.1	3
Sample 5	0.35	0.08	22.0	6
Sample 6	0.43	0.05	12.6	13
Sample 7	0.39	0.08	19.4	14
Sample 8	0.31	0.02	7.4	2
Sample 9	0.40	0.08	19.5	6
Sample 10	0.42	0.10	24.2	4
Sample 11	0.36	0.08	21.7	5
Sample 12	0.40	0.06	14.3	19

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Vitamin B12 Analysis in Slim Fast type Dietary Beverages by Biosensor Method

Parameters	Reg. Slim Fast	Spike Amount	Expected	Obtained	% Recovery
Mean mcg/100 mL (n=3)	1.02	0.48	1.50	1.52	101.7
SD	0.095			0.044	8.4
% RSD	9.3			2.9	8.3
Micro Results (n=2) mcg/100 mL	0.97				
HPLC Result (n=6) mcg/100 mL	0.98				

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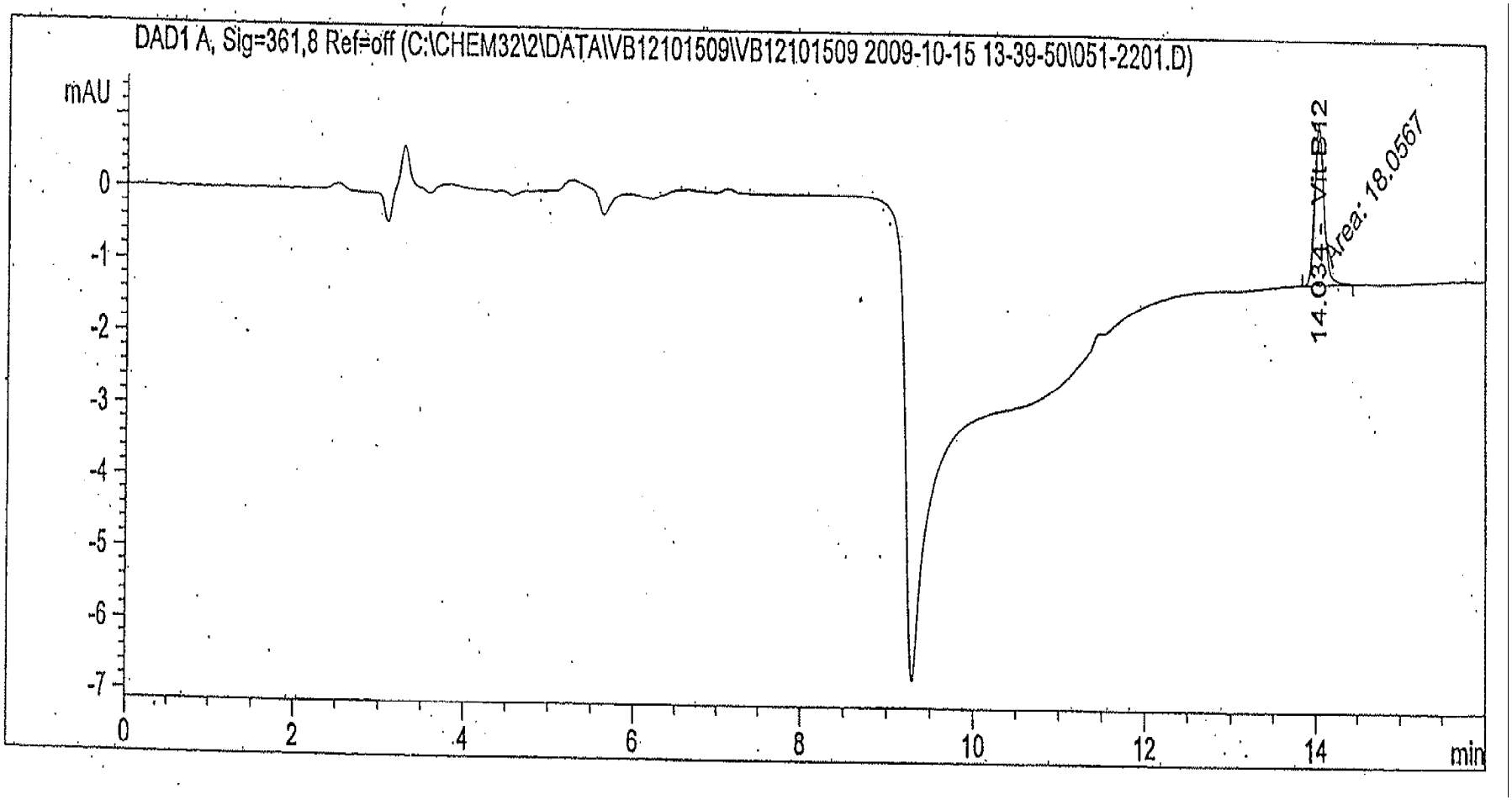
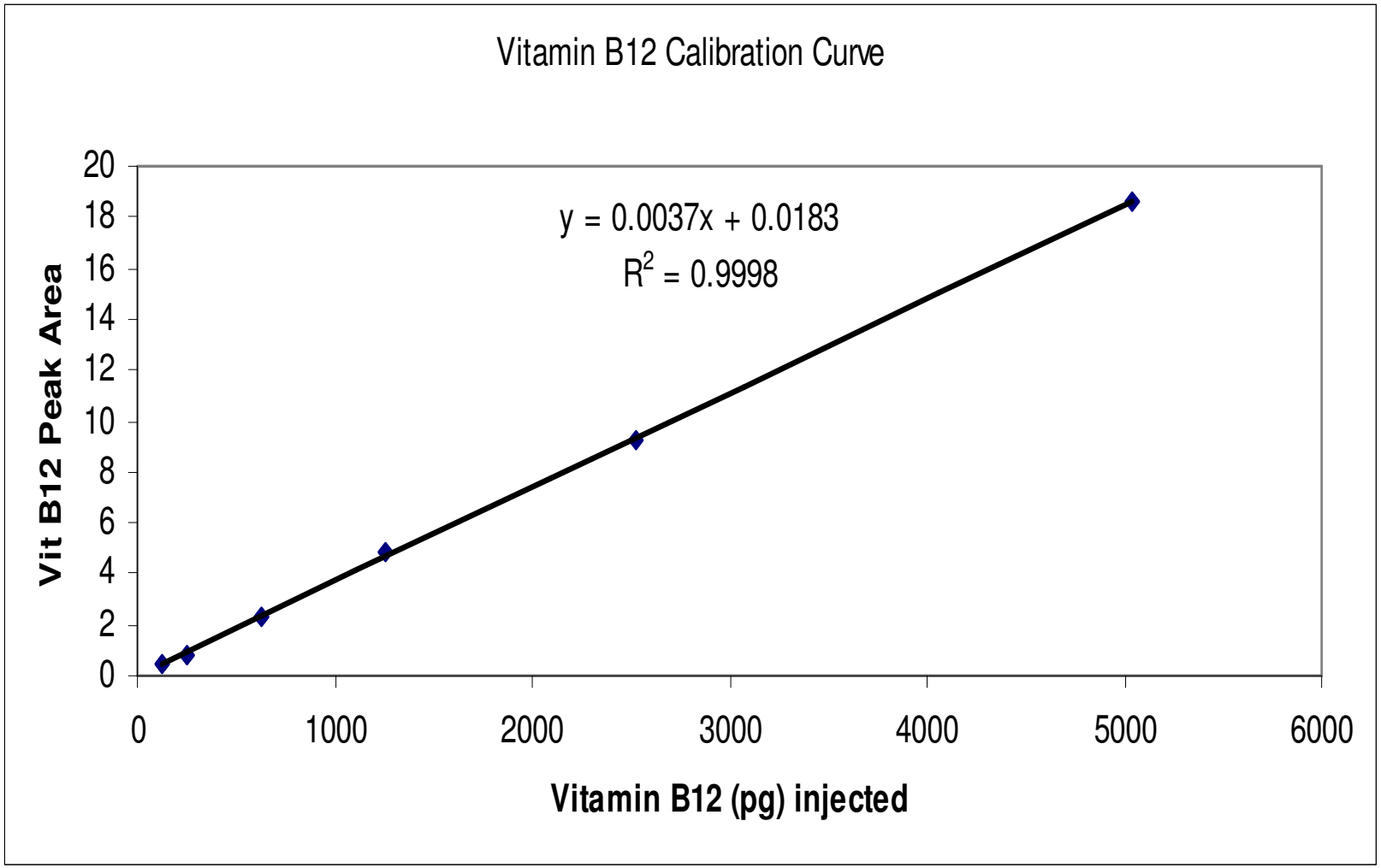
Vitamin B12 Chromatogram



Vitamin B12 Analysis by HPLC – Calibration Curve



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HPLC Analysis - Recovery of standard through the immuno-affinity column

Pramameter	Vitamin B12 ng/mL		HPLC results
	HPLC	Expected	% of expected
Mean (n=3)	42.5	49.0	86.7
S.D.	0.17	-	0.36
% RSD	0.4	-	0.4

10 mL of 49 ng/mL standard passed throgh the immunoaffinity to check the recovery.

Analysis performed using R-Biopharm protocols.

Vitamin B12 by HPLC in NIST SRM 1846 (Infant Formula dairy based powder) – Different Extraction

Extraction Treatment	Buffer	Vitamin B12 mcg/g		
		Repl 1	Repl 2	Expected
Vortex 1 min and Shake 30 min	Sodium Acetate	0.031	0.027	**0.039**
Vortex 1 min and Shake 30 min	Phosphate Citarte	0.02	0.022	
Pressure Cooker 30 min	Phosphate Citarte	0.035	0.033	
Microwave 30 min	Phosphate Citarte	0.026	0.027	

Vitamin B12 by HPLC in NIST SRM 1846 (Infant Formula dairy based powder) – Precision & Accuracy

Pramameter	Vitamin B12 mcg/g		HPLC results
	HPLC	Expected	% of expected
Mean (n=9)	0.037	0.039	94.9
S.D.	0.001	-	-
% RSD	3.3	-	-
Spike Studies			
Spike mcg/g	HPLC mcg/g	Expected mcg/g	% Recovery
0.117	0.154	0.155	100.6
0.060	0.097	0.097	100.0

Extraction using enzyme treatment per R-Biopharm protocols
Extraction solution contained cyanide.
Results of Spike analysis by HPLC are mean of triplicates at at 0.117 mcg/g and of 9 analyses at 0.060 mcg/100g levels.

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Vitamin B12 by HPLC – in Crystal Light Energy Beverages by different methods

Enegy Beverage Sample	Vitamin B12 (mcg/100 mL)					
	Expected	Micro	ELISA	Biacore*	VitaFast	HPLC**
Sample 1	0.31	0.54	0.25	0.31	0.54	
Sample 2	0.31	0.38	0.24	0.33	0.48	
Sample 3	0.31	0.42	0.36	0.37	0.52	
Sample 4	0.31	0.37	0.24	0.32	0.43	0.32
Sample 5	0.31	0.32	0.38	0.32	0.40	
Sample 6	0.31	0.37	0.26	0.37	0.46	
Sample 7	0.31	0.33	0.28	0.33	0.41	
Sample 8	0.31	0.40	0.30	0.33	0.54	
Sample 9	0.31	0.31	0.18	0.27	0.39	0.33
Sample 10	0.31	0.34	0.23	0.25	0.41	0.32
Sample 11	0.31	0.34	0.29	0.32	0.41	
Sample 12	0.31	0.34	0.39	0.33	0.41	
Mean	**0.31**	**0.37**	**0.28**	**0.32**	**0.45**	**0.32**
SD		*0.06*	*0.06*	*0.04*	*0.06*	*0.01*
%RSD		16.8	22.9	11.1	12.8	1.8
% of Micro			***76***	***86***	***121***	***87***
Expected TOT		2 weeks	1 day	2 days	3-4 days	2 days

*Average of two trials; **Using immunoaffinity clean-up and concentration.
HPLC Results are average of two trials.

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Vitamin B12 by HPLC – in Crystal Light Energy Beverages by different methods

Enegy Beverage Sample	Vitamin B12 (mcg/100 mL)				
	HPLC (n=3)	Micro	ELISA	Biacore*	VitaFast
Mean (n =12)	**0.32**	**0.37**	**0.28**	**0.32**	**0.45**
SD	*0.01*	*0.06*	*0.06*	*0.04*	*0.06*
%RSD	1.8	16.8	22.9	11.1	12.8
% of Micro	87.0		***76***	***86***	***121***
Expected TOT	2 days	2 weeks	1 day	2 days	3-4 days

*Average of two trials

Expected amount = 0.31 mcg/100 mL

Vitamin B12 by HPLC – in Crystal Light Energy Wild Strawberry Beverages

Sample Lot #	Vitamin B12 (ng/mL)		
	HPLC*[a]	Micro	Expected
1	6.1	5.5	2.4
2	5.8	4.9	2.3
3	4.1	3.9	2.4
4	4.9	4.3	2.4
5	5.1	6.2	2.5

*Mean of 3 independent analyses;

HPLC[a] - samples purified and concentrated using immunoaffinity column and analyzed by HPLC.

Vitamin B12 by HPLC and other methods in different Beverages

Sample	ID	Vitamin B12 mcg/100 mL in Beverage samples			
		HPLC		Biosensor	Micro
		a	b		
Energy Beverages	Flavor A	0.28	0.35	0.36	0.37
	Flavor B	0.31	0.34	0.47	0.31
	Flavor C	0.35	0.31	0.47	0.34
Snapple	Rescue	0.27	NA	NA	0.27*

*- Label Value; NA = Not analyzed

Vitamin B12 by HPLC in Crystal Light Energy Beverages - Precision

Parameters	Vitamin B12 mcg/100 mL		
	HPLC Method	Mico Method	Label Value
Mean (n=6)	0.35	0.34*	0.31
SD	0.03	-	-
% RSD	7.9	-	-

* Mean of two trials

Vitamin B12 by HPLC in Crystal Light Energy Beverages – Spike Recovery

Spike Level ng/mL	ng/mL	SD	%RSD	% Recovery	Micro Results ng/mL
0	4.06	0.29	7.2		4.1*
2.4	6.26	0.39	6.2	96.9	
4.6	8.23	0.47	5.7	95.2	
12	17.47	1.5	8.6	108.8	

HPLC results are based on 6 independent analyses.

*-Micro results are mean of two analyses.

Vitamin B12 by HPLC in Orange Tang Beverage Sample – Precision and accuracy

Pramameter	Vitamin B12 mcg/100 mL		
	HPLC	Expected	Micro Results
Mean (n=6)	<0.01	0.25	<0.1
Spike Studies			
Spike mcg/100mL	HPLC mcg/100mL	Expected mcg/100mL	% Recovery
0.49	0.383	0.49	78.2
0.98	0.828	0.98	84.5

Spike Results mean of 3 trials.

Vitamin B12 by HPLC in an intense red 6x concentrated energy Beverage Sample – Precision and accuracy

Pramameter	Vitamin B12 mcg/100 mL		
	HPLC	Label	% of Expected
Mean (n=3)	1.979	1.910	103.6
SD	0.068	-	-
% RSD	3.4	-	-
Spike Studies			
Spike mcg/100mL	HPLC mcg/100mL	Expected mcg/100mL	% Recovery
5.665	6.893	7.644	90.2

Spike Results mean of 3 trials.

Vitamin B12 by HPLC in dairy based dietary supplement beverage (Slim Fast) - Precision

Pramameter	Vitamin B12 mcg/ 100g		HPLC results	Label Value
	HPLC	Micro Results	% of Micro	mcg/100g
Mean (n=6)	0.98	0.97	100.6	0.65
S.D.	0.20	-	-	-
% RSD	20.9	-	-	-

Extraction using enzyme treatment per R-Biopharm protocols

Extraction solution contained cyanide.

Microbiological assay Results are mean of duplicates.

Vitamin B12 by HPLC in a fortified breakfast Cereal Sample - Precision

Pramameter	Vitamin B12 mcg/100 g		HPLC results
	HPLC	Micro Results	% of Micro
Mean (n=6)	18.8	18.0	104.4
S.D.	0.56	-	-
% RSD	3.0	-	-

Micro results mean of analysis in duplicate.

Vitamin B12 by HPLC in multigrain flakes Cereal Sample - Precision

Parameters	Vitamin B12 mcg/100 mL		
	HPLC Method	Mico Method	Label Value
Mean (n=13)	16.4	16.5*	12.5
SD	0.40	-	-
% RSD	2.4	-	-

* Mean of two trials

Vitamin B12 Analysis using LC-MS/MS API 3200™ - System Settings

- **Positive electrospray using Turbo V source**
- **Dwell time is 100 mS**
- **First MRM 1transition used for quantification**
- **Second MRM 2 transition available for ion ratio use**
  - MRM 1 / MRM 2

Period 1

Analyte	Q1	Q3
Thiamine 1	265	122
Thiamine 2	265	144
Pyridoxine 1	170	134
Pyridoxine 2	170	152
Nicotinamide 1	123	80
Nicotinic Acid 1	124	80
Pantothenate 1	220	202
Pantothenate 2	220	184

Period 2

Analyte	Q1	Q3
Vit B12 1	678	147
Vit B12 2	678	359
Biotin 1	245	227
Biotin 1	245	97
Folic Acid 1	442	295
Folic Acid 2	442	176
Riboflavin 1	377	243
Riboflavin 2	377	198

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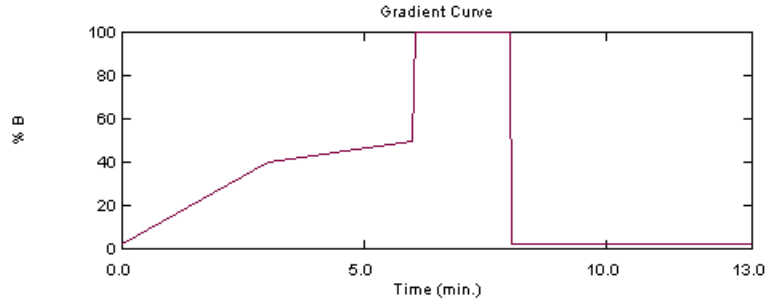
Liquid Chromatography Acquisition Method

- **Shimadzu Prominence binary gradient system with autosampler & column oven**
- **Data System:** **Cliquid® Quant 2.0 Software with Analyst® 1.5 Software**
- **Column:** **Restek Ultra Aqueous C18, 4.6 x 50 mm, 3 µm (Col Vol ~830 µL)**
- **MP A:** **0.05% formic acid**
- **MP B:** **0.05% formic acid in methanol**
- **Flow Rate:** **1000 µL/min** **BP <2500 psi**
- **Temp:** **40°C**
- **Gradient:**

Min	%A	%B
0	98	2
3	60	40
6	50	50
6.05	0	100
8	0	100
8.05	98	2
13	98	2



- **Inj Vol:** **50 µL for Vitamin B12 and other vitamins <10 ng/mL concentrations** and 10 µL for routine analysis of > 10 ng/mL concentration

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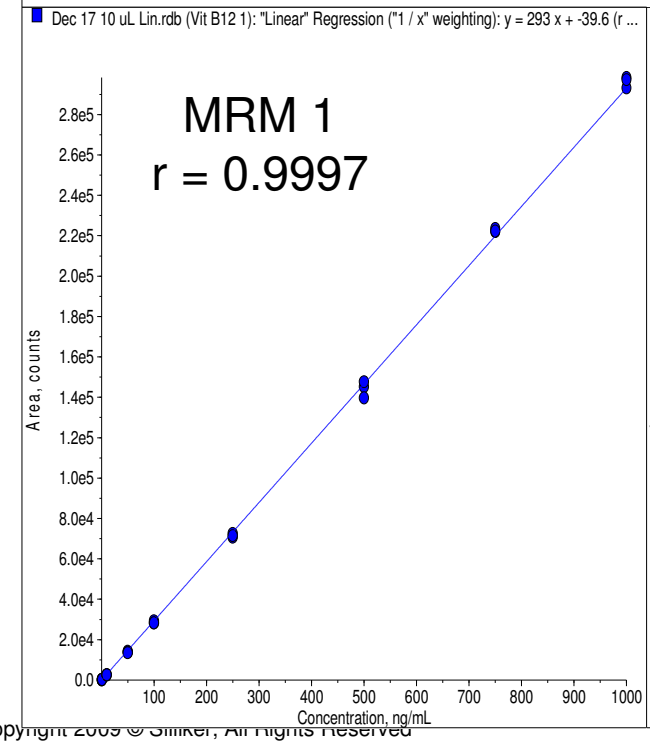
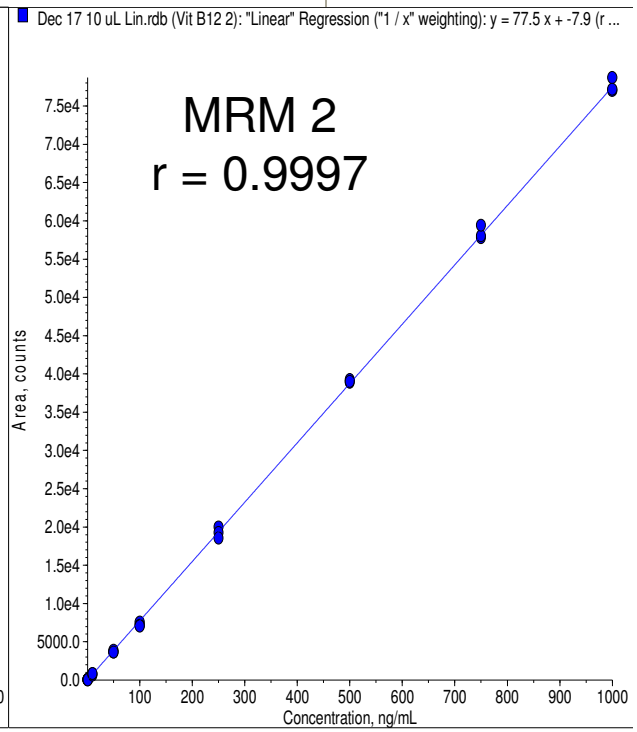
Vitamin B12 Linearity and Ion Ratio

	Sample Name	Analyte Peak Name	Analyte Retention Time (min)	Analyte Peak Area (counts)	Analyte Concentration (ng/mL)	Calculated Concentration	Accuracy (%)
6	Std 2	Vit B12 1	3.71	2.73e+002	1.00	1.07	107.
7	Std 3	Vit B12 1	3.71	2.69e+003	10.0	9.29	92.9
8	Std 3	Vit B12 1	3.70	2.45e+003	10.0	8.48	84.8
9	Std 3	Vit B12 1	3.71	2.56e+003	10.0	8.87	88.7
10	STd 4	Vit B12 1	3.71	1.42e+004	50.0	48.5	96.9

0.5 -1000 ng/mL





Sample Name	Ion Ratio
Std 1	2.67
Std 1	4.00
Std 1	3.53
Std 2	4.50
Std 2	2.10
Std 2	9.95
Std 3	4.07
Std 3	2.98
Std 3	3.06
STd 4	3.90
Std 4	3.57
Std 4	3.69
Std 5	3.88
Std 5	3.94
Std 5	4.01
Std 6	3.63
Std 6	3.67
Std 6	3.86
Std 7	3.73
Std 7	3.56
Std 7	3.79
Std 8	3.84
Std 8	3.85
Std 8	3.74
Std 9	3.81
Std 9	3.79
Std 9	3.85

Ave	3.794
Std Dev	0.260
%RSD	6.859
High Range	4.575
Low Range	3.014

Vitamin Linearity and LOD Summary Using 10 µL Injection

	10 uL Injection		ng/mL		
Vitamin	**Lin Range**	**r Value**	**LOD**	**IR Low**	**IR High**
Pyridoxine	0.5- 1000	0.9845	<0.1	0.892	1.046
Vit B12	0.5 - 1000	0.9997	0.2	3.014	4.575
Folic Acid	0.5 - 1000	0.9996	0.2	1.647	2.388
Nicotinamide	0.5 - 1000	0.9942	0.1		
Biotin	1 - 1000	0.9993	0.9	3.817	4.861
Pantothenic Acid	1 - 1000	0.9993	0.3	0.851	1.177
Thiamine	0.5 -1000	0.9991	<0.1	3.875	5.178
Riboflavin	10 - 1000	0.9991	5.4	2.083	3.544

Ion Ratio (IR) Range = Ave ± 3(Std Dev)

9 Stds from 0.5 – 1000 ng/mL, triplicate injection at each concentration

Energy Beverage Sample Data

Energy Beverage				Data as ng/mL				
	Nicotinamide	Pyridoxine	Pantothenic Acid	Thiamine	Folic Acid	Biotin	Vit B12	Riboflavin
Label Value		800	4000			120	2.5	
Inj 1	9580	1110	1740	15.5	40	109		11.4
Inj 2	9480	1150	1810	13.0	32.5	106	3.75	7.12
Inj 3	9500	1200	1810	17.7	25.7	103	3.75	8.14
Ave	**9520.0**	**1153.3**	**1786.7**	**15.4**	**32.7**	**106.0**	**3.8**	**8.9**
Std Dev	2.4	45.1	40.4	2.4	7.2	3.0	0.0	2.2
%RSD	0.02	3.9	2.3	15.3	21.9	2.8	0.0	25.2
Ion Ratio		**0.981**	**0.974**	**4.803**	**2.283**	**4.243**	**4.050**	**2.477**
IR High		1.046	1.177	4.861	2.388	4.861	4.575	3.544
IR Low		0.892	0.851	3.817	1.647	3.817	3.014	2.083

Ion Ration Range

Ion Ratio = Confirmed

Vitamin B12 Analysis by LC-MS/MS and conventional microbiological methods in different beverage samples

Results Micro or LC-MS	Triple Quad Model	Vit B12 ng/mL	
		Expected	Obtained
Milk based Nutrition Formula			
Micro (AOAC) Results	-	0.78	0.7
LC-MS Appl. Biosyst.	API 3200*		1.5
Nutrient Enhanced Water			
Micro (AOAC) Results	-	5.0	13.0
LC-MS Appl. Biosyst.	API 3200		6.5
LC-MS ThermoFisher	TSQ Access**		5.2
Energy Beverage			
Micro (AOAC) Results	-	2.5	6.2
LC-MS Appl. Biosyst.	API 3200		3.8
LC-MS Varian	Varian 320-MS***		2.6

*using Shimadzu Prominence with Restek Aq C18 column (n=3).

**using Accela HPLC with Hypersil Gold Phenyl column, gradient 0.1% aquoeous formic acid to 0.1% formic acid in methanol (n=3).

***using Varian HPLC with Varian Pursuit C18 column, gradient 0.1% aquoeous formic acid to 0.1% formic acid in methanol (n=3).

Micro (AOAC) Results are mean of duplicates analyses.

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Conclusions

- Different methods of vitamin B12 analysis were compared for their accuracy in selected beverage samples. Methods included conventional microbiological, Biosensor method, VitaFast micro assay, ELISA assay and HPLC analysis using immunoaffinity purification and concentration.
- The accuracy of the Biosensor and the HPLC assays were established by accurate analysis of the NIST SRM 1846 (Infant Formula).
- The Biosensor and HPLC methods provided results similar to the AOAC micro method in various energy beverage samples as well as the dietary beverage Slim Fast samples. The results were precise. Both methods provided satisfactory spike recovery.

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Conclusions

- In a comparative study beverage samples were analyzed by different methods. ELISA, HPLC and Biosensor method provided similar results. Values were practically similar to those obtained by the Micro method. The VitaFast results were slightly on higher side and the ELISA on the lower side.
- The HPLC analysis of vitamin B12 in cereal samples was found to be precise and accurate (values similar to the Micro assay).
- LC-MS method was investigated. The method is linear in a broad range. Three beverage samples were analyzed using different MS. Values provided by different LC-MS methods were closer to each other than the micro method. Studies are in progress to further optimize the MS method.

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